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*FELLOWSHIP FINAL REPORT*

# Evolution of innate immunity at biomineralized barriers

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REPORT INFO

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*Host scientist:* **Sophie Réhault-Godbert**
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ABSTRACT

In oviparous animals such as birds, embryonic development occurs in the egg, and after oviposition there is no further possibility of material exchange with the hen to fulfill the needs of the embryo. In such a context, the egg must contain all systems required for proper development of a living organism. Among these, the chorioallantoic membrane (CAM) is a novel placenta-like structure which is the nexus for many different physiological and metabolic processes including acid-base balance, breathing and calcium solubilization from the eggshell that is re-allocated to assist bone and tissue formation in the embryo. Moreover, it is believed to play a pivotal role in innate immunity to protect the embryo, in close interaction with the eggshell and the eggshell membranes. Therefore, weakening of the eggshell during CAM-mediated decalcification is hypothesized to be compensated by upregulation of innate immune mechanisms. In order to characterize this role of the CAM during embryonic development, we performed transcriptomics, proteomics and bioinformatics analyses. This residence was also the opportunity to stimulate a new international dynamic collaboration towards investigating innate immunity in diverse biomineralized structures (shells, bone, corals).

## 1- Introduction

The calcareous egg of birds and reptiles, and previously dinosaurs, is a successful reproductive adaptation to the desiccating terrestrial environment. Embryonic development within this autonomous chamber has been shaped through evolution to resist physical and pathogenic challenges, while satisfying the metabolic and nutritional needs of the developing embryo. The evolution of the oviparous reproduction model to the viviparous model has led to important distinctions between corresponding extra-embryonic structures, particularly concerning the placenta. In humans, for example, the allantoic sac is not an independent structure as in the avian embryo, but forms part of the umbilical cord. The urine of the human embryo is therefore secreted directly into the amniotic sac whereas the chicken embryo secretes metabolic waste into the allantoic sac, thus forming the AIF (allantoic fluid) (Bellairs and Osmond, 2014). However, the important presence of proteins and peptides in connection with the immune response and defense in human AmF (amniotic fluid) is mirrored by the demonstration of such molecules in the avian AIF and AmF. In contrast, much less is known about the specific molecules that intervene in the mobilization of calcium (decalcification) from the avian ES (Chien et al, 2009), and the potential upregulation of innate immune genes at this critical site.

The chorioallantoic membrane (CAM) is three-layered structure that embraces both the embryo and all associated extra-embryonic structures (Gabrielli and Accilly, 2010). It possesses many physiological functions and constitutes the first barrier against ES-penetrating pathogens. However, the molecular players associated with

these functions remain largely unknown. Transcriptomic and proteomic studies of the developmental changes that occur in the CAM will be fruitful, particularly if augmented with functional studies of cellular and molecular changes. We believe that the CAM (which can be likened to the mammalian placenta) has many unexplored functions and plays a major role in the development and protection of the living avian embryo.

The first step of the project was to perform a literature review describing the progressive transformation of egg innate immunity by embryo-generated structures and mechanisms over the 21-day course of egg incubation (Hincke et al, 2019). This article became the basis for a chapter for the 3rd edition of the Avian Immunology textbook (Réhault-Godbert, et al., 2021), and stimulated the organization of a symposium in 2021 dedicated to comparative immunology between different biomineralized organisms (Hincke and Rehault-Godbert, 2021). As an outcome of this symposium, we proposed a research topic on this theme, as co-editors, to the journal "Frontiers in Immunology" (Hincke et al., 2022). Four articles are published under the framework of this topic, to date, including two of which M. Hincke and S. Réhault-Godbert are co-authors (Kulshreshtha et al., 2022; Moreau et al., 2022). During this residency, we were successful in obtaining financial support to recruit a doctoral student to this project, and thus co-supervised Maeva Halgrain during her research on this project (October, 2019 – September, 2022).

## 2- Experimental details

Two experimental approaches were undertaken.

**Experiment #1.** We assessed eggshell demineralization and calcification of the embryo skeleton after 12 and 16 days of incubation, and analyzed the expression of several candidate genes in the CAM by q-RT-PCR: carbonic anhydrases that are likely involved in secretion of protons for eggshell dissolution (CA2, CA4, CA9, Gabrielli et al., 2001), ions transporters and regulators (CALB1, SLC4A1, ATP6V1B2, SGK1, SCGN, PKD2) and vitamin-D binding protein (GC). In this experiment, we also measured eggshell weight, thickness, and strength, and concomitant calcification of the embryonic skeletal system (alcian blue and red alizarin staining).

Experiment #2. We focused on two developmental stages: 11 and 15 days of incubation, corresponding to the immature and mature stages of CAM development, respectively. We explored the simultaneous morphological modifications in the eggshell (ES), eggshell membrane (ESM) and the CAM at both stages by scanning electron microscopy. In parallel, we performed RNA sequencing of the CAM to investigate the regulation of its gene expression during incubation while a functional annotation of overexpressed genes was performed to improve our knowledge of the physiological functions of the CAM.

## 3- Results and discussion

*Eggshell decalcification during incubation parallels skeletal mineralization of the embryo and a specific expression profile in the chorioallantoic membrane*

From the first experiment, our results confirmed that eggshell weight, thickness, and strength decreased during incubation, with a concomitant increase in calcification of embryonic skeletal system. In the CAM, the expression of CA2 increased during incubation while CA4 and CA9 were expressed at similar levels at both stages. SCL4A1 and SCGN were expressed, but not differentially, between the two stages, while the expression of ATP6V1B2 and PKD2 genes decreased. The expression of SGK1 and TRPV6 increased over time, although the expression of the latter gene was barely detectable. In parallel, we analyzed the expression of these candidate genes in the yolk sac (YS), which mediates the transfer of yolk minerals to the embryo during the first half of incubation. In YS, CA2 expression increases during incubation, similar to the CAM, while the expression of the other candidate genes decreases. Moreover, CALB1 and GC genes were found to be expressed during incubation in the YS, in contrast to the CAM where no expression of either was detected. This first

study demonstrated that the regulation of genes involved in the mobilization of egg minerals during embryonic development is different between the YS and CAM extraembryonic structures (Figure 1). It also revealed the limits of using the candidate gene approach as the expression of many candidates was low and/or not regulated during CAM maturation. These data were published in Poultry Science in 2022 (M. Halgrain et al., 2022).

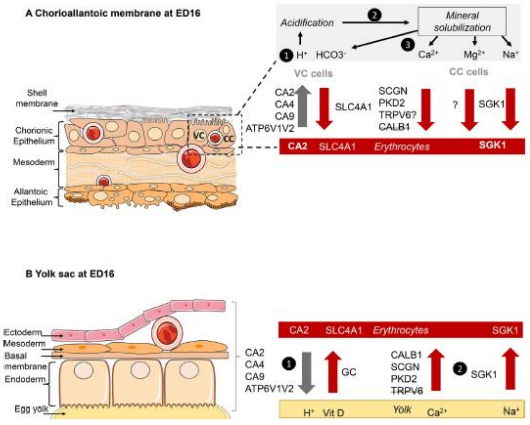


**Figure 1.** Hypothetical representation of the role of candidate genes in the chorionic epithelium of the CAM (A) and in the YS (B) during the second half of incubation, in mineral mobilization from the eggshell and the yolk, respectively. For a complete description, see Halgrain et al., 2022a.

*During incubation, the CAM undergoes major structural changes that accompany decalcification of the inner eggshell.*

We explored the simultaneous morphological modifications in the eggshell (ES), eggshell membranes (ESM) and the CAM at 11 and 15 days of incubation by scanning electron microscopy. We observed that the tips of the mammillary knobs of the ES remain tightly attached to the ESM fibers, while their bases become progressively eroded and then detached from the bulk ES (Figure 2).

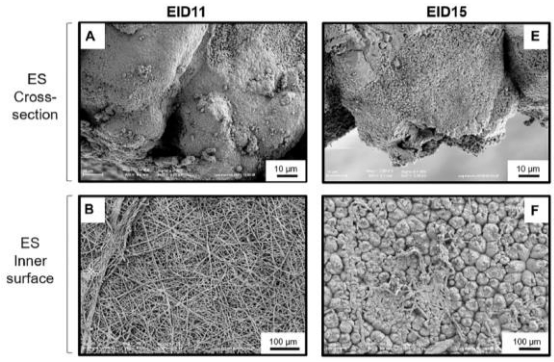


**Figure 2.** SEM analyses of the structural modifications of the ES and ESM between 11 and 15 days of incubation (EID11 and EID15, respectively). For a complete description, see Halgrain et al., 2022b).

Concomitantly, the CAM undergoes major structural changes as highlighted previously (Makanya et al., 2016) that include the progressive differentiation of villous cells whose villi extend to reach the ESM and the ES.

Eggshell decalcification and weakening during incubation is likely to impair the ability of the ES to protect the embryo. It is assumed that the CAM could counteract this apparent weakening as an additional layer of physical, cellular and molecular barriers against environmental perturbations, including pathogen aggression, dehydration and physical shock. To address this question, we analyzed in parallel the expression profile of the CAM at these two stages.

*The functional annotation of genes overexpressed after 15 days of incubation (versus 11 days of incubation) highlights a crucial role of the CAM in mineral ion metabolism and transport, and immunity functions*

Results from RNA sequencing showed that CAM cellular maturation coincides with the overexpression of 4225 genes.

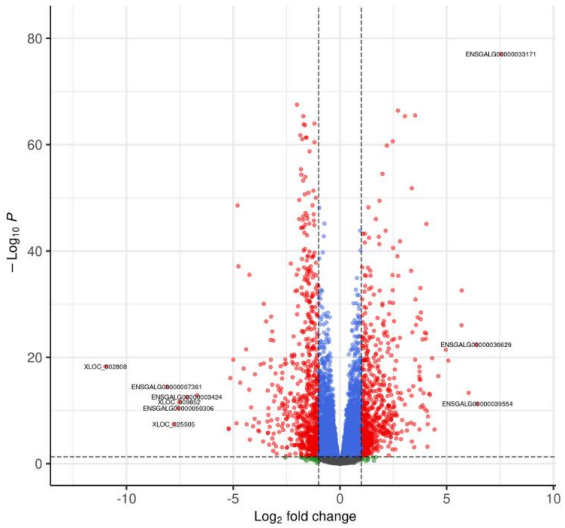


**Figure 3.** Volcano plot illustrating differentially expressed genes between EID15 (right) *versus* EID11 (left) (red dots). Personal communication, © INRAE, C. Hennequet-Antier.

Many genes that are overexpressed at 15 days of incubation encode proteins involved in mineral metabolism, innate immunity and to a lesser extent homeostasis, angiogenesis, reproduction and regulation of hypoxia. The identification of new functional gene candidates opens new perspectives of research in the field of developmental biology and cancer research, as the CAM is a model biomaterial for the study of angiogenesis (Moreno-Jiménez et al., 2017) and the testing of biomaterials (Valdes et al., 2002).

These results will be submitted for publication by the end of 2022.

## 4- Conclusion

Collectively, our results provide a molecular basis to further investigate the cellular and molecular mechanisms accompanying the role of the CAM in the decalcification of the eggshell to provide minerals for mineralizing the embryo skeleton, and allowed the identification of additional molecular candidates implicated in the regulation of innate immunity during embryonic development. To go further in the study of this latter CAM function, we believe that microbial stimulation is likely to reveal yet unknown cellular and molecular processes that may be of major interest towards our understanding of innate immunity of the fertilized egg during embryonic development.

During this residency, Prof. M. Hincke also had the opportunity to collaborate with other members of the host team focusing on the eggshell, which resulted in the publication of five articles and reviews (Stapane et al., 2019, 2020; Le Roy et al., 2019, 2021) and one book chapter (Nys et al. 2021).

## 5- Perspectives of future collaborations with the host laboratory

Our first original results have revealed the importance of the chorioallantoic membrane in different aspects of embryo development. New ideas have emerged while we are still analysing the most recent data related to the role of the CAM in innate immunity which protects the embryo throughout its development. As an extension of the Studium symposium organized in 2021, Maxwell Hincke and S. Réhault-Godbert are in charge of organizing a specific session dedicated to "Immunity in biomineralized barriers" for the 17th International Symposium on Biomineralization that will be held in Saint-Etienne, France in 2023 (08/28 - 09/01/2023), which is expected to stimulate new collaborations with international partners.

To continue our active collaboration, Prof. Maxwell Hincke has currently applied for a sabbatical stay in the host laboratory in 2024, which will be an excellent opportunity to develop new collaborative projects between INRAe, France and the University of Ottawa, Canada.

## 6- Articles published in the framework of the fellowship

Gautron, J., Stapane, L., Le Roy, N., Nys, Y., Rodriguez-Navarro, A.B. and Hincke, M.T. (2021) Avian eggshell biomineralization: An update on its structure, mineralogy and protein tool kit. BMC Molecular and Cell Biology. 22: 11.

Halgrain, M., Bernardet, N., Crepeau, M., Même, N., Narcy, A., Hincke, M., and Réhault-Godbert, S. (2022a) Eggshell decalcification

and skeletal mineralization during chicken embryonic development: defining candidate genes in the chorioallantoic membrane. Poultry Science 101: 101622.

Halgrain, M., Georgeault, S., Bernardet, N., Hincke, M.T. and Réhault-Godbert, S. (2022b) Concomitant morphological modifications of the avian eggshell, eggshell membranes and the chorioallantoic membrane during embryonic development. Frontiers in Physiology, section Avian Physiology 13:838013.

Halgrain, M., Bernardet, N., Hennequet-Antier, C., Hincke, M., and Réhault-Godbert, S. (2022c) Expression profiling of the active chick embryo chorioallantoic membrane identifies genes functioning in ion transport and innate immunity. (Genomics, to be submitted).

Hincke, M.T., Da Silva, M., Guyot, N., Gautron, J., McKee, M.D., Guabiraba, R. and Réhault-Godbert, S. (2019) Dynamics of Structural Barriers and Innate Immune Components during Incubation of the Avian Egg: Critical Interplay between Autonomous Embryonic Development and Maternal Anticipation. Journal of Innate Immunity. 11: 111-124.

Kulshreshtha, G., D'Alba, L., Dunn, I.C., Rodriguez-Navarro, A.B., Rehault-Godbert, S. and Hincke, M.T. (2022) Properties, genetics and innate immune function of the cuticle in egg-laying species. Frontiers in Immunology 13: 838525.

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Nys, Y., Gautron, J., Rodriguez-Navarro, A.B., Hincke, M. T. (2021) Mechanisms and hormonal regulation of shell formation: supply of ionic and organic precursors, shell mineralization. Chapter 32, pp 833-879. In : *Sturkie's Avian Physiology*, 7th Edition. (Eds. C. Scanes and S. Dridi) Elsevier.

Réhault-Godbert, S., Guyot, N., Gautron, J., Guabiraba, R. and Hincke, M.T. (2021) Immune function and defense mechanisms in the egg and embryo. Chapter 13, pp 365-386. In: *Avian Immunology*, 3rd Edition (Eds. T. Göbel, L. Vervelde, T. Schat and B. Kaspers).

Stapane, L., Le Roy, N., Hincke M.T. and Gautron, J. (2019) The glycoproteins EDIL3 and MFGE8 regulate vesicle - mediated eggshell calcification in a new model for avian biomineralization. Journal of Biological Chemistry 294(40): 14526-14545.

Stapane, L., Le Roy, N., Ezagal, J., Rodriguez-Navarro, A.B., Labas, V., Combes-Soia, L., Hincke, M.T. and Gautron, J. (2020) Avian eggshell formation reveals a new paradigm for vertebrate mineralization via vesicular amorphous calcium carbonate. Journal of Biological Chemistry, 295(47): 15853-15869.

**Invited presentations by Hincke (2018-2022):**

UMR Biologie des Oiseaux et Aviculture (BOA) reunion. (INRA-Tours, Nouzilly, France; March 20, 2018). "Innate Immunity at Biomineralized Barriers".

L'OEuf : du symbole à l'assiette. (Le Pôle Alimentation de l'université de Tours, Tours, France ; March 22, 2019). « Transformation d'une ressource précieuse: la coquille d'œuf. »

Le Studium Jeudi (Loire Valley Institute for Advanced Studies, April 4, 2019). "Critical Features of Innate Immunity at Biomineralized Barriers".

UMR Biologie des Oiseaux et Aviculture (BOA) reunion. (INRA-Tours, Nouzilly, France; May 20, 2019). “Immunité innée à une barrière biominéralisée - la membrane chorioallantoïque”.

Le Studium and University of Tours, France; Community of international researchers. (March 12, 2020). “Internationalization at the Faculty of Medicine, University of Ottawa”

Virtual Conference: Innate immunity in a biomineralized context: trade-offs or synergies? (March 23-24, 2021) “The Chorioallantoic Membrane: Insight from Proteomics.”

Le Studium Jeudi (Loire Valley Institute for Advanced Studies, April 7, 2022). “Update on Innate Immunity at Biomineralized Barriers”.

UMR Biologie des Oiseaux et Aviculture (BOA) reunion. (INRAE-Tours, Nouzilly, France; May 23, 2022). “L’Immunité innée intégrée aux barrières biominéralisées: le modèle CAM”.

## 7- Acknowledgements

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